PERSISTENT ANTIGENIC VARIATION OF INFLUENZA A VIRUSES AFTER INCOMPLETE NEUTRALIZATION IN OVO WITH HETEROLOGOUS IMMUNE SERUM

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Antigenic differences between strains of influenza viruses first were demonstrated (1) shortly after the discovery of the agents. A large amount of study has been directed toward the extent and the significance of such differences (2–7). The bulk of the evidence indicates that strains obtained from a single epidemic do not differ strikingly (5, 8–10) but that strains recovered from epidemics occurring in different years may show major antigenic differences (11–13). Both the extent of the differences in antigenic composition and their bearing upon immunization against influenza have become increasingly evident since 1947 when so called influenza A prime strains (14) first were recovered and vaccines prepared with earlier strains failed to give immunity in man (15).

Not only do various strains of influenza virus show antigenic differences as early as they can be tested after recovery from patients, but also a single strain may undergo antigenic variation during passage in laboratory hosts. Early work (16–18) indicated that antigenic variation may occur when strains are adapted to a new host. Later studies (19–21) provided strong evidence in support of this conclusion. There is now good reason to suspect that prolonged passage of influenza virus in any new species may lead to the development of antigenic variation. Passage of two initially similar egg strains in mice has led to divergent changes in their antigenic patterns (19).

Although the evidence for the occurrence of antigenic variation with influenza viruses is impressive, there is a lack of evidence as to the mechanism by which such variants arise and become dominant. In line with current concepts of mutation in microbial species, it might be assumed that naturally occurring variants of viruses are selected by chance on passage in unnatural hosts. This hypothesis has been offered as an explanation for the variations observed on passage of influenza virus in the mouse lung (19). Although a similar hypothesis might be put forward for the different strains recovered from man, an alternative requires consideration. Multiplication of influenza

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viruses in normal laboratory animals occurs in the absence of any induced immunity against the agents; circulating antibodies are not present. In man, particularly in adults, this is generally not the case and as a result of prior infections with the agents a measure of induced immunity is present; circulating antibodies are demonstrable in a high proportion of instances (22). Under these circumstances, if antigenic variants emerge during multiplication, the possibility arises that those least neutralizable by the antibodies present would be most likely to infect other susceptible cells. Thus, in persons with different infective backgrounds or with different immunization experiences who become infected with a single strain, there could appear viral populations with different antigenic patterns. This possibility has been considered previously (10).

The hypothesis was subjected to experimental test in a laboratory model in the following manner: Different strains of influenza virus were passed in series in the chick embryo in the presence of immune serum against other strains. The quantity of serum was adjusted so that the virus was partially but not completely neutralized during each passage. The evidence obtained in this study indicates that antigenic variants emerge regularly under these conditions. The data indicate also that antigenic variation did not occur during limited serial passage in a single host species in the absence of immune serum. In addition, evidence was assembled which suggests that the antigenic pattern of the variant differs from that of the parent virus in a manner predictable in terms of the immune serum used and that the variant virus retains its altered antigenic pattern after further serial passage in the absence of immune serum.

Materials and Methods

Viruses.—Four strains of influenza A virus were used: one, PR8, has been through many mouse lung and chick embryo passages; the others, SH, 965, and FM1, have not received mouse lung passages and have been through relatively few chick embryo passages: 5, 6, and 10, respectively. 965 (10) was received from Dr. R. M. Taylor, International Health Division, The Rockefeller Foundation, and was recovered from a patient ill in November, 1943. SH was recovered in this laboratory from a patient ill in March, 1948. PR8 (20) and FM1 (11) are well known strains which have been used by numerous workers. Infected allantoic fluid was used as a source of virus; between experiments fluids were stored at −70°C. Virus dilutions were prepared in broth containing 10 per cent inactivated normal horse serum. In the experimental section the lines of a particular strain (*e.g.*, FM1) are identified by subscripts as follows: original strain = $FM1_o$; control passage strain = $FM1_c$; heterologous serum passage strain = $FM1_s$ (subscript corresponding to the heterologous strain, *i.e.*, SH).

Virus Titrations.—Virus infectivity titrations were done by the intra-allantoic technique described by Hirst (23). Hemagglutination titrations were done by the technique described by Hirst (24) using a final concentration of 0.5 per cent chicken RBC.

Immune Serum.—Antiviral serum was prepared in rabbits as previously (25). Each was given 10 cc. of infected allantoic fluid intravenously and 1 week later received an additional 10 cc. intraperitoneally. Serum was obtained 2 to 3 weeks after the second injection and was

stored at 4°C. without preservative. Immune serum was prepared against each of the 4 viruses before the experiments were begun. Additional immune serum was prepared after each of the viruses had been subjected to control serial passage in chick embryos as well as after serial passage in the presence of heterologous immune serum. In each instance 2 rabbits were immunized. For hemagglutination-inhibition titrations and *in ovo* neutralization titrations, serum was inactivated at 56°C. for 30 minutes. In the experimental section the various immune sera against a particular strain (*e.g.*, FM1) are identified by subscripts as follows: original antiserum = $FM1_o$; control passage antiserum = $FM1_c$; antiserum prepared after passage with heterologous serum = $FM1_s$ (subscript corresponding to the heterologous strain, *i.e.*, SH).

Neutralizing Antibody Titrations.—*In ovo* neutralization titrations were carried out with twofold dilutions of inactivated serum and a constant amount of virus by the technique described previously (25). A 10^{-4} dilution of infected allantoic fluid, corresponding to 10^3 to 10^4 infective doses of virus, was employed. It has been shown that the experimental error of neutralizing antibody titrations carried out in this manner is no greater than that of hemagglutination-inhibiting antibody titrations (25). Pooled serum from 2 immune rabbits was used in each titration unless otherwise stated. Cross-neutralization titrations with 2 or more strains and corresponding antisera were, in so far as feasible, carried out simultaneously.

Hemagglutination-Inhibiting Antibody Titrations.—These were carried out in a manner similar to that described by Hirst (24); the detailed procedure is given in a preceding paper (25). Final concentrations of 0.5 per cent chicken RBC and 8 hemagglutination units of virus were used. Pooled serum from 2 immune rabbits was used in each titration unless otherwise stated. Cross-hemagglutination-inhibition titrations with 2 or more strains and corresponding antisera were regularly carried out simultaneously.

Antibody Titer Ratios.—The practice of Burnet and Lush (26), as modified by Hirst (5), of expressing the titration results obtained with a particular serum in terms of the ratio of the heterologous titer divided by the homologous titer was employed to facilitate interpretations of the results. The so called "avidity factor" employed by Hirst (5) was not used because it was desired to make direct comparisons between results obtained by neutralization and hemagglutination-inhibition titrations, and there appears to be no justification for using an empirical correction factor with neutralization titrations. The method used for the analysis of the results, which is similar to that employed by Smith and Andrewes (4), makes such a correction factor unnecessary. The geometric mean of the ratio (r_1), found on dividing the heterologous titer obtained with virus 2 by the homologous titer obtained with virus 1, and the ratio (r_2), found on dividing the heterologous titer obtained with virus 1 by the homologous titer obtained with virus 2, is given by the function: $r = \sqrt{r_1 \times r_2}$. The value r gives in a single figure the extent of the antigenic difference between 2 viruses when both agents and both antisera are used in a cross-serological reaction. It is evident that when handled in this manner a ratio greater than 1 (*e.g.*, $r_1 = 2$) tends to cancel out with a corresponding ratio smaller than 1 (*e.g.*, $r_2 = \frac{1}{2}$) and when both are equal in extent yield a value for $r = 1$, indicating no antigenic difference. Because the ratio r is the geometric mean of the two ratios obtained with the heterologous viruses and the homologous sera, it yields a value which gives equal weight to differences found in either direction, *i.e.*, serum 1 *versus* virus 2 and serum 2 *versus* virus 1, and makes it unnecessary to assume that antigenic differences should be reciprocal. In the various figures presented below, the ratio r is used in the form $1/r$ in order to simplify the data. As is evident, the homologous titer ratio is by definition 1 in all instances.

Serial Passages of Virus.—A 10^{-2} dilution of allantoic fluid infected with one strain was mixed with each of a series of twofold dilutions of inactivated immune serum against another strain. The mixtures were held at room temperature for 10 to 15 minutes and then 0.2 cc.

of each was injected into the allantoic sac; 3 embryos were used for each mixture. Allantoic fluid was harvested after 48 hours' incubation at 35°C. and tested by the hemagglutination technique. Positive fluids from embryos which had received the highest concentration of immune serum were used for passage. At each passage a 10^{-2} dilution of allantoic fluid, unless otherwise stated in the experimental section, and a series of twofold dilutions of immune serum were employed. After the desired number, 6 to 12, of serial passages in the presence of immune serum was completed, one additional allantoic passage without serum was made as routine. The only exception was with the SH-965 series. Infected fluid from the last passage was stored at −70°C. as a source of virus for antibody titrations and aliquots were used for immunization of rabbits as described above. In every instance a parallel control series of passages of equal number was carried out in which no immune serum was used. Infected

TABLE I

Cross-Hemagglutination-Inhibition Reactions with 4 Strains of Influenza A Virus

Serum	Virus*			
	FM1	SH	PR8	965
	Serum inhibition titer‡			
FM1	**640**	512	160	40
SH	810	**320**	20	40
PR8	160	64	**2560**	240
965	320	160	320	**1280**
	Titer ratio§			
FM1	1	1/1.2	1/4	1/16
SH	2.5	1	1/16	1/8
PR8	1/16	1/32	1	1/10.7
965	1/4	1/8	1/4	1

* 8 units final of each virus.

‡ Each titer represents the geometric mean of 3 or more end-points.

§ Titer ratio = heterologous titer divided by homologous titer.

allantoic fluid from the last passage of each control series also was stored at −70°C. as a source of virus for antibody titrations and aliquots also were used for immunization of rabbits.

EXPERIMENTAL

Antigenic Relations between Original Strains.—The hypothesis which led to this study was as follows: Influenza viral populations are inhomogeneous as a result of the presence of antigenic variants which arise by chance in small numbers. Such variants can be selected by procedures designed to separate them from the far larger number of typical viral particles. The objective of the investigation was to discover if the antigenic pattern of a strain undergoes alteration on passage in the chick embryo in the presence of immune serum against a different strain adjusted so as to cause only partial neutralization of the inoculum. Four viruses were selected; 2 (PR8 and 965) are typical influ-

enza A viruses which were recovered in 1934 and 1943, respectively; 2 others (FM1 and SH) are so called influenza A prime viruses which were recovered in 1947 and 1948, respectively. All but PR8 were recovered first in chick embryos and had been carried through only 5 to 10 passages in this host. With the exception of PR8, none of the strains had ever been passed in the mouse lung.

The antigenic relations between the 4 strains were determined by serological tests. Cross-hemagglutination-inhibition reactions were carried out by the methods described above and the results are shown in Table I. By the *in vitro* procedure there was no indication of a difference between FM1 and SH

TABLE II

Cross-Neutralization Reactions with 4 Strains of Influenza A Virus

Serum	Virus*			
	FM1	SH	PR8	965
	Serum neutralizing titer‡			
FM1	**320**	95	40	5
SH	46	**80**	0	2
PR8	5	2	**2560**	260
965	4	3	40	**160**
	Titer ratio			
FM1	1	1/3.4	1/8	1/64
SH	1/1.7	1	1/80	1/40
PR8	1/512	1/1280	1	1/9.8
965	1/40	1/53	1/4	1

* 10^{-4} dilution of allanotic fluid.

‡ Serial twofold dilutions of inactivated serum were employed.

as is evident from the various titer ratios. By means of the ratio r, $(r = \sqrt{r_1 \times r_2})$, as described above, the extent of the cross-relation between any 2 strains, as indicated by the 2 heterologous titer ratios, can be expressed in a single figure. With FM1 and SH, $r = 1.4$, indicating no significant antigenic difference between them. A definite difference was found between the antigenic patterns of PR8 and 965; $r = 1/6.5$. Even more marked differences were revealed when FM1 was compared with 965; $r = 1/8$. Equally definite differences emerged when SH was related to 965; $r = 1/8$. It is important to reiterate that FM1, SH, and 965 had been passed only in the chick embryo. Thus, it appears that there are clear differences between strains of influenza A virus which have not been subjected to laboratory passage in a mammalian host.

Cross-neutralization reactions with the 4 strains were carried out *in ovo* by

the methods described above and the results are shown in Table II. Much more striking antigenic differences between the strains were revealed by neutralization than by hemagglutination-inhibition. This becomes evident

TABLE III

Cross-Hemagglutination-Inhibition Reactions with Original and Control Embryo Passage Strains

Serum	Virus*							
	FM1		SH		PR8		965	
	o	c‡	o	c‡	o	c§	o	c§
	Serum inhibition titer‖							
FM1 o	**640**	1280	512	320				
" c‡		**640**		810				
SH o	810	910	**320**	250				
" c‡		2560		**640**				
PR8 o					**2560**	1820	240	160
" c§						**2560**		160
965 o					320	640	**1280**	1620
" c§						640		**2040**
	Titer ratio							
FM1 o	1	2	1/1.2	1/2				
" c		1		1.3				
SH o	2.5	2.8	1	1/1.3				
" c		4		1				
PR8 o					1	1/1.4	1/10.7	1/16
" c						1		1/16
965 o					1/4	1/2	1	1.2
" c						1/3.2		1

* 8 units final each virus. o = original; c = control passage.

‡ After 12 embryo passages.

§ " 6 " "

‖ Each titer represents the geometric mean of 2 or more end-points.

when the various titer ratios are compared. The difference between FM1 and SH is indicated by $r = 1/2.4$; between PR8 and 965 by $r = 1/6.3$; between FM1 and 965 by $r = 1/50.6$; and between SH and 965 by $r = 1/46$. These results provide additional confirmation for the view (10–13) that strains of influenza A virus obtained in different years and carried through only a few

passages in the chick embryo may reveal striking differences in antigenic patterns.

Antigenic Relations between Original and Control Passage Strains.—Before attempting to interpret changes which resulted from chick embryo passage in the presence of heterologous immune serum, it was essential to discover if any demonstrable antigenic alterations occurred on control passage of the strains in the chick embryo in the absence of serum. Both FM1 and SH were subjected to 12 serial passages in the chick embryo; PR8 and 965 were carried

TABLE IV

Cross-Neutralization Reactions with Original and Control Embryo Passage Strains

Serum	Virus*			
	PR8		965	
	o	c‡	o	c‡
	Serum neutralizing titer			
PR8 o	2560	2560	260	320
" c‡		320		20
965 o	40	20	160	160
" c‡		80		640
	Titer ratio			
PR8 o	1	1	1/9.8	1/8
" c		1		1/8
965 o	1/4	1/8	1	1
" c		1/8		1

* 10^{-4} dilution each virus.

‡ After 6 embryo passages.

through 6 serial passages in this species. In these control experiments immune serum was not employed. The procedure used was identical with that described above and immune sera were prepared with the original strains as well as with each of the strains after completion of the control passages.

Antigenic relations between the original and control passage strains were determined by serological tests. The results of cross-hemagglutination-inhibition reactions are shown in Table III. In no instance did the original (subscript o) and the control passage (subscript c) lines show evidence of significant antigenic differences. This is most clearly evident when the titer ratios obtained with each of the strains and their corresponding immune sera are compared. Even when antisera against heterologous strains, *i.e.*, SH *versus* FM1, or 965 *versus* PR8, were employed, no evidence was found that antigenic

differences had arisen as a result of 6 to 12 serial passages in the chick embryo in the absence of immune serum.

The result of cross-neutralization reactions with the original (o) and control passage (c) lines derived from PR8 and 965 are shown in Table IV. As with the *in vitro* reactions, there was close correspondence between the titer ratios found with each of the original and control lines. This provides additional evidence in support of the conclusion that no change in the antigenic pattern was occasioned by limited passage in the chick embryo itself. On the basis of this evidence, it seemed justifiable to consider that significant alterations in antigenic pattern which appeared after passage in the presence of heterologous immune serum were attributable to the antiviral serum.

Reduction in Neutralizability of Strains on Passage in the Presence of Heterologous Immune Serum.—Eight series of serial passages were carried out in which dilutions of heterologous immune sera were mixed with a strain prior to each passage. Each of the 4 strains was employed in one or more of the series; the experimental conditions are described above. A condensed summary of the results is shown in Table V. In the first 6 series a constant amount of virus, *i.e.* 10^{-2}, was employed with twofold dilutions of immune serum; in the last 2 series, as little as a 10^{-7} dilution of virus was used in order to obtain some neutralization of the heterologous strain. Increasing amounts of the same immune serum were required to neutralize a constant amount of each virus as the serial passages were continued in the first 6 series. In the case of the last 2 series with SH and 965, complete neutralization was not obtained even when high concentrations of serum, *i.e.* 1:2, and very small amounts of virus, 10^{-7}, were employed. The results show that there was in the first 6 series a progressive decrease in the neutralizability of the virus by heterologous immune serum and provide an indication that the antigenic patterns of the strains were undergoing alteration as the passages continued. Direct tests of this postulate are recorded below.

Antigenic Variation with SH and FM1 on Passage with Heterologous Immune Serum.—Two separate and complete experiments, in each of which 12 serial passages were carried out, were undertaken with SH and FM1. In the first experiment one pair of heterologous immune sera was used; in the second another pair of similar immune sera was employed. The procedures used and the controls employed are described above.

The results of cross-neutralization reactions with SH, FM1, and variants derived from each on passage in the presence of heterologous immune serum are shown in Table VI. From the titer ratios it is evident that SH_f, the strain derived from SH on passage with anti-FM1 serum, differed from the parent virus (SH). Evidence for an antigenic difference was obtained after both independent experiments. Similarly, the titer ratios reveal that $FM1_s$, the variant derived from FM1 on passage with anti-SH serum, showed marked

differences in antigenic pattern from the parent virus (FM1). The antigenic differences between the 2 parent strains and the variants derived from them,

TABLE V

Decrease in Virus Neutralization on Serial Passage in Presence of Heterologous Immune Serum

Virus		Serum*	Passages in presence of serum	Dilution of serum required to neutralize virus	
	Dil.				Change
			No.		*fold*
FM1	10^{-2}	SH (*a*)	0	20	
			6	10	
			12	<5	>4
SH	"	FM1 (*a*)	0	40	
			6	40	
			12	10	4
FM1	"	SH (*b*)	0	20	
			6	5	
			12	<5	>4
SH	"	FM1 (*b*)	0	80	
			6	40	
			12	40	2
PR8	"	965	0	40	
			3	10	
			6	<2	>20
965	"	PR8	0	80	
			3	5	
			6	<2	>40
SH	"	965	0	<2	
	10^{-7}		6	<2	
	"		12	<2	0
965	10^{-2}	SH	0	<2	
	10^{-7}		6	<2	
	"		12	<2	0

* *a* and *b* indicate sera from different pairs of rabbits.

as determined by *in ovo* neutralization reactions, are depicted graphically in Fig. 1.

The ratios (r) represent the geometric means of the 2 heterologous titer ratios (r_1 and r_2) obtained with any 2 strains and their antisera. As described above, the ratio is computed by

means of the equation $r = \sqrt{r_1 \times r_2}$, and gives in a single figure the extent of the dissimilarity in the antigenic patterns of 2 strains. It is noteworthy that, in each case, the ratio r is dependent upon a minimum of 4 antibody titrations. Under the conditions of these experiments, values of $1/r$ of 2 or more may be considered significant. The larger the value of r, the greater is the antigenic dissimilarity. It can be shown that, on a percentage basis, a value

TABLE VI

Cross-Neutralization Reactions with SH, FM1, and Variants Derived from Each on Passage with Heterologous Immune Serum

Experiment	Serum*	Virus*			
		SH_c	SH_f	$FM1_c$	$FM1_s$
		Serum neutralizing titer			
I	SH_o	**160**	20	40	0
	SH_f	80	**160**	160	5
	$FM1_o$	80	40	**320**	20
	$FM1_s$	5	5	40	**160**
		Titer ratio			
	SH_o	1	1/8	1/4	1/160
	SH_f	1/2	1	1	1/32
	$FM1_o$	1/4	1/8	1	1/16
	$FM1_s$	1/32	1/32	1/4	1
		Serum neutralizing titer			
II	SH_o	**160**	20	40	0
	SH_f	80	**40**	160	10
	$FM1_o$	80	40	**320**	20
	$FM1_s$	5	2	40	**80**
		Titer ratio			
	SH_o	1	1/8	1/4	1/160
	SH_f	2	1	4	1/4
	$FM1_o$	1/4	1/8	1	1/16
	$FM1_s$	1/16	1/40	1/2	1

* Subscript o = original; c = after control passage; f = after passage with FM1 serum; s = after passage with SH serum.

of 2 for $1/r$ is equivalent to an antigenic cross-relationship of 50 per cent; 4 = 25 per cent; 8 = 12.5 per cent; 16 = 6.2 per cent; 32 = 3.1 per cent; 64 = 1.6 per cent; 128 = 0.8 per cent; etc.

The variant derived from SH (SH_f) and the variant derived from FM1 ($FM1_s$) were different in antigenic pattern from the parent viruses which had been subjected to parallel control passages of equal duration. Closely similar results regarding the antigenic variations induced by this procedure were

evident both in Experiments I and II, indicating that the alterations were reproducible under the experimental conditions employed.

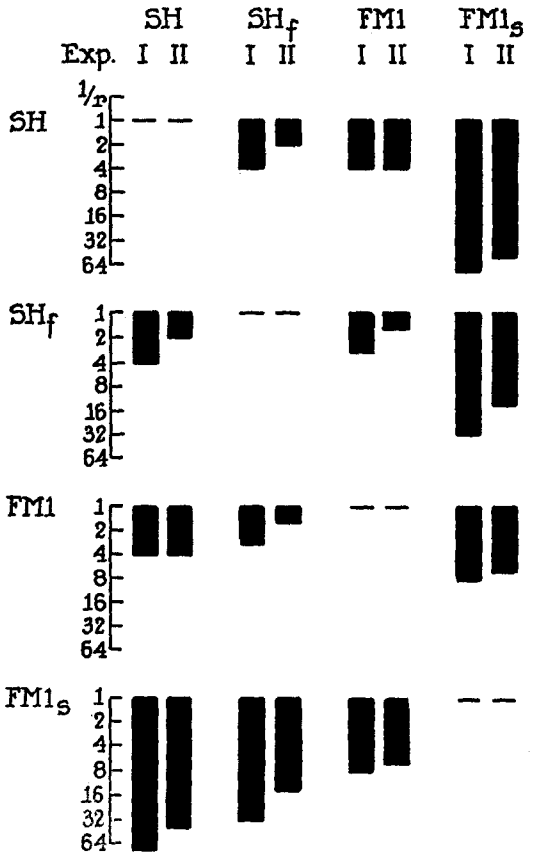

FIG. 1. Extent of *in ovo* cross-neutralization reactions with SH, FM1, and variants derived from them on serial passage *in ovo* in presence of heterologous immune serum. Experiments I and II were identical but independent. The ratio r, shown as the reciprocal, is the geometric mean of the 2 heterologous titer ratios and represents the degree of antigenic dissimilarity between any 2 strains. The larger the value of $1/r$, the greater is the difference in the antigenic patterns of 2 strains. Homologous ratios are by definition equal to 1. Serum titers and titer ratios are shown in Table VI.

Cross-hemagglutination-inhibition reactions between SH, FM1, and the variants derived from them on passage with heterologous immune serum are shown in Table VII. From the titer ratios obtained it is obvious that the differences between SH_f and SH_c revealed by cross-neutralization reactions *in ovo* were not apparent by cross-hemagglutination-inhibition reactions after either Experiment I or II. The antigenic differences between $FM1_s$ and $FM1_c$,

however, were of an extent closely similar to those found by *in vivo* reactions. The differences in the antigenic patterns between the parent strains and their variants, as shown by hemagglutination-inhibition reactions, are shown graphically in Fig. 2. The ratio *r* employed is identical with that used in Fig. 1. It is

TABLE VII

Cross-Hemagglutination-Inhibition Reactions with SH, FM1, and Variants Derived from Each on Passage with Heterologous Immune Serum

Experiment	Serum*	Virus*			
		SH_o	SH_f	$FM1_o$	$FM1_s$
		Serum inhibition titer			
I	SH_o	**320**	160	640	40
	SH_f	640	**240**	1280	320
	$FM1_o$	320	160	**1280**	160
	$FM1_s$	320	60	320	**1280**
		Titer ratio			
	SH_o	1	1/2	2	1/8
	SH_f	2.6	1	5.3	1/1.3
	$FM1_o$	1/4	1/8	1	1/8
	$FM1_s$	1/4	1/21.3	1/4	1
		Serum inhibition titer			
II	SH_o	**640**	320	2560	80
	SH_f	640	**320**	1280	640
	$FM1_o$	320	320	**2560**	320
	$FM1_s$	320	80	320	**1280**
		Titer ratio			
	SH_o	1	1/2	4	1/8
	SH_f	2	1	4	2
	$FM1_o$	1/8	1/8	1	1/8
	$FM1_s$	1/4	1/16	1/4	1

* Symbols are identical with those used in Table VI.

clear that the *in vitro* reactions, although revealing somewhat less marked differences than the *in vivo* reactions, showed that the variant $FM1_s$ derived from FM1 was markedly different from the parent virus which had been subjected to parallel control serial passage. Of importance is the additional evidence that the results obtained in the two separate experiments were closely similar indicating a high degree of reproducibility.

The data shown in both Figs. 1 and 2 demonstrate that the variant $FM1_s$ differed from SH to a much greater degree than the parent FM1. Because the

variant was obtained on passage with anti-SH, such an alteration in antigenic pattern was to be expected in terms of the postulate stated above. It appears clear that prolonged passage of either SH or FM1 in the presence of antiserum against the other resulted in each of the two experiments in the emergence of variant viral strains possessing antigenic patterns different from those of the parent strains. Because closely similar differences were found after passage with either of the 2 pairs of immune sera employed, it seems that the antigenic pattern of the variant virus is reproducible to a surprising degree.

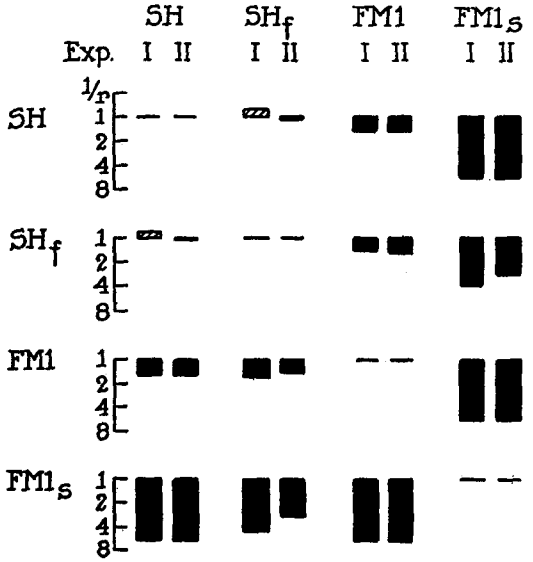

FIG. 2. Extent of cross-hemagglutination-inhibition reactions with SH, FM1, and variants derived from them on serial passage *in ovo* in presence of heterologous immune serum. Serum titers and titer ratios are shown in Table VII.

Antigenic Variation with PR8 and 965 on Passage with Heterologous Immune Serum.—Two successive experiments, consisting of 6 serial passages each, were carried out with PR8 and 965. In the first experiment each virus was passed in the presence of immune serum against the other. Following this experiment additional passages were carried out without serum employing the strains obtained from the first experiment. The objective was to discover if antigenic differences emerging in the first experiment would persist during a second passage series in the absence of an environment experimentally arranged to select variants of different antigenic pattern. The experimental procedure was identical with that described above and included, as in experiments with the other viruses, parallel series of control passages without serum.

Results of cross-neutralization reactions with PR8 and 965, as well as the

variants derived from them in the two successive experiments, are shown in Table VIII. From the titer ratios it is evident that there was some antigenic difference between $PR8_c$ and $PR8_9$, the variant obtained from PR8 by passage in the presence of anti-965 serum; the difference was shown more clearly by

TABLE VIII

Cross-Neutralization Reactions with PR8, 965, and Variants Derived from Each on Passage with Heterologous Immune Serum

Experiment	Serum*	Virus*			
		$PR8_o$	$PR8_9$	965_c	965_p
		Serum neutralizing titer			
I	$PR8_o$	**2560**	1280	320	160
With serum	$PR8_9$	1280	**320**	2	0
	965_o	20	5	**160**	40
	965_p	160	10	160	**320**
		Titer ratio			
	$PR8_o$	1	1/2	1/8	1/16
	$PR8_9$	4	1	1/160	1/320
	965_o	1/8	1/32	1	1/4
	965_p	1/2	1/32	1/2	1
		Serum neutralizing titer			
II	$PR8_o$	**2560**	1280	320	160
Additional passages	$PR8_9$	1280	**160**	2	2
without serum	965_o	20	5	**160**	40
	965_p	160	10	160	**160**
		Titer ratio			
	$PR8_o$	1	1/2	1/8	1/16
	$PR8_9$	8	1	1/80	1/80
	965_o	1/8	1/32	1	1/4
	965_p	1	1/16	1	1

* Subscript o = original; c = after control passage; 9 = after passage with 965; p = after passage with PR8 serum.

the reactions with heterologous immune sera, *i.e.*, 965_o and 965_p. Clear differences in antigenic pattern were revealed between 965_c and 965_p, the variant derived from 965 on passage in the presence of anti-PR8 serum. The antigenic differences appeared during serial passages carried out with heterologous immune serum in the first experiment; of more importance, they persisted during serial passages in the second experiment when no immune serum was employed. The results after conversion to the ratio r by means of the equation given above are shown in Fig. 3. The extent of the differences between strains

is indicated by the length of the blocks. The fact that variants arose and persisted for at least 6 passages in the absence of serum is evident from the results obtained both with homologous immune sera and with heterologous immune sera.

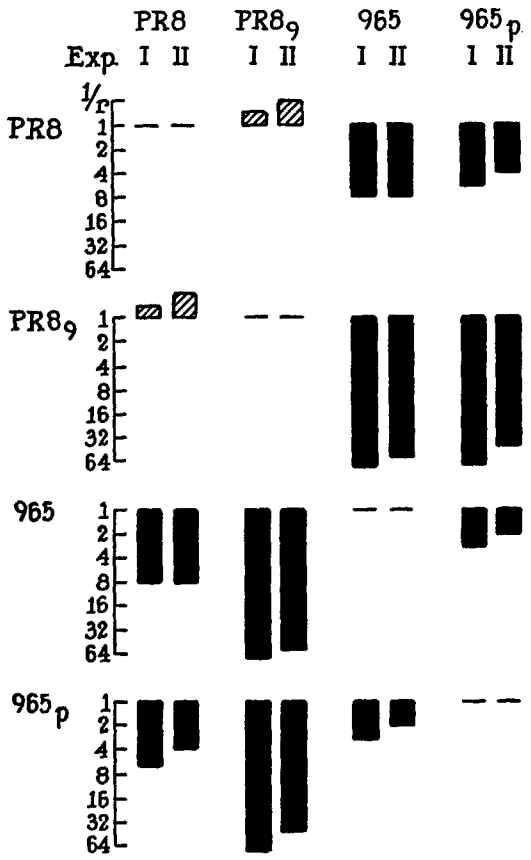

FIG. 3. Extent of *in ovo* cross-neutralization reactions with PR8, 965, and variants derived from them on serial passage *in ovo* in presence of heterologous immune serum. Experiment I, with immune serum; Experiment II, additional passages without serum. Serum titers and titer ratios are shown in Table VIII.

The results of cross-hemagglutination-inhibition reactions with PR8, 965, and variants derived from them are shown in Table IX. With this pair of viruses, in contrast to SH and FM1, the extent of the antigenic differences appears to be more evident from the hemagglutination-inhibition reactions *in vitro* than from the neutralization reactions *in vivo*. The finding with PR8 and 965, as well as with SH and FM1, that *in vitro* and *in vivo* cross-serological

reactions were not strictly comparable, either qualitatively or quantitatively, is consistent with the evidence obtained earlier (25) indicating that hemagglutination-inhibiting and neutralizing antibodies against influenza viruses are not identical. That the variant strain $PR8_9$ was different from the parent, PR8,

TABLE IX

Cross-Hemagglutination-Inhibition Reactions with PR8, 965, and Variants Derived from Each on Passage with Heterologous Immune Serum

Experiment	Serum*	Virus*			
		$PR8_c$	$PR8_9$	965_c	965_p
		Serum inhibition titer			
I	$PR8_o$	**2560**	320	320	40
With serum	$PR8_9$	2560	**1280**	80	40
	965_o	320	80	**2560**	160
	965_p	320	160	640	**2560**
		Titer ratio			
	$PR8_o$	1	1/8	1/8	1/64
	$PR8_9$	2	1	1/16	1/32
	965_o	1/8	1/32	1	1/16
	965_p	1/8	1/16	1/4	1
		Serum inhibition titer			
II	$PR8_o$	**2560**	320	320	40
Additional passages	$PR8_9$	5120	**5120**	80	160
without serum	965_o	320	80	**2560**	160
	965_p	1280	160	1280	**5120**
		Titer ratio			
	$PR8_o$	1	1/8	1/8	1/64
	$PR8_9$	1	1	1/64	1/32
	965_o	1/8	1/32	1	1/16
	965_p	1/4	1/32	1/4	1

* Symbols are identical with those used in Table VIII.

and that the variant 965_p was different from the parent, 965, seems evident. Again, as in the neutralization reactions, the differences are clearly seen with heterologous sera, *e.g.*, $PR8_c$ and $PR8_9$ *versus* serum 965_o. These results are presented graphically in Fig. 4. The evidence indicates that the variant strains obtained in the first experiment were antigenically dissimilar to the parent strains and reacted less well with heterologous immune sera used to select them than did the parent viruses. Such a change in antigenic pattern was to be expected in terms of the original hypothesis.

The changes induced in PR8 indicate that antigenic variants can be obtained even from a very old laboratory strain (20) which has been carried through hundreds of passages in various host species. The persistence of the altered antigenic patterns of the variants through additional passages in the absence of immune serum is clearly indicated by the close similarity in the results

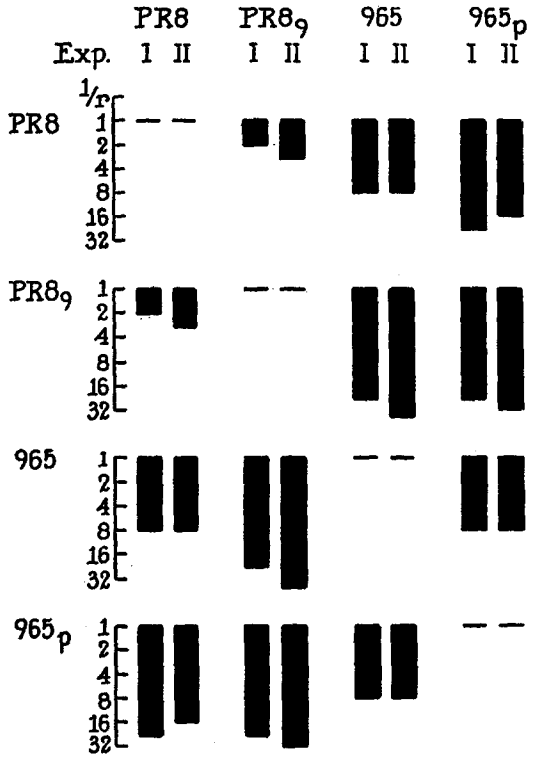

FIG. 4. Extent of cross-hemagglutination-inhibition reactions with PR8, 965, and variants derived from them on serial passage *in ovo* in the presence of heterologous immune serum. Serum titers and titer ratios are shown in Table IX.

obtained in the first and succeeding experiments. The evidence suggests that, once a new antigenic pattern has emerged, it may remain as a transmissible character in the absence of a selective environment.

Antigenic Patterns of SH and 965 after Passage with Heterologous Immune Serum.—Experiments similar to those described above also were carried out with SH and 965. This pair of viruses was chosen because they were so markedly different both in cross-neutralization and cross-hemagglutination-inhibition reactions as is evident from the results presented in Tables I and II. The objective was to discover if antigenic variants could be obtained from widely

different strains by the procedure employed with strains of closer relation. Twelve serial passages with each virus were carried out in the presence of immune serum against the other. The experimental conditions and the control passages were identical with those employed in the preceding experiments with the exception that after the first few passages very small inocula, *i.e.* 10^{-7}, were mixed with undiluted immune serum in an effort to obtain partial neutralization.

The results of cross-neutralization and cross-hemagglutination-inhibition reactions with SH, 965, and the substrains obtained from them are shown in

TABLE X

Cross-Serological Reactions with SH, 965, and Substrains Obtained on Passage with Heterologous Immune Serum

Serum*	Virus*			
	SH_c	SH_9	965_c	965_8
	Serum neutralizing titer			
SH_c	**40**	80	0	0
965_c	2	0	**160**	160
	Titer ratio			
SH_c	1	2	1/40	1/40
965_c	1/80	1/160	1	1
	Serum inhibition titer			
SH_c	**160**	160	40	20
965_c	160	40	**1280**	640
	Titer ratio			
SH_c	1	1	1/4	1/8
965_c	1/8	1/32	1	1/2

* Symbols identical with those used in other tables.

Table X. In this experiment immune sera were not prepared against either of the strains after they had been passed in the presence of heterologous antisera. This was thought to be unnecessary when the titer ratios indicated that there was but little evidence of an antigenic difference between either substrain and the corresponding parent strains. Results after computation of the ratio r by the function given above are presented graphically in Fig. 5. It is clear that there was no significant difference between SH and SH_9, the substrain derived from SH after passage in the presence of anti-965 serum. There was, however, some suggestion of a difference in antigenic pattern between 965 and 965_8, the substrain derived from 965 on passage in the presence of anti-SH

serum. Because this difference was evident only in hemagglutination-inhibition reactions and immune sera against the substrains were not available for confirmatory cross-serological reactions, it is doubtful that any significant evidence of antigenic variation was obtained with SH and 965. With strains as widely different as SH and 965, which fail to show significant evidence of cross-neutralization *in ovo*, it would not be anticipated that antigenic variants should emerge under the experimental conditions employed.

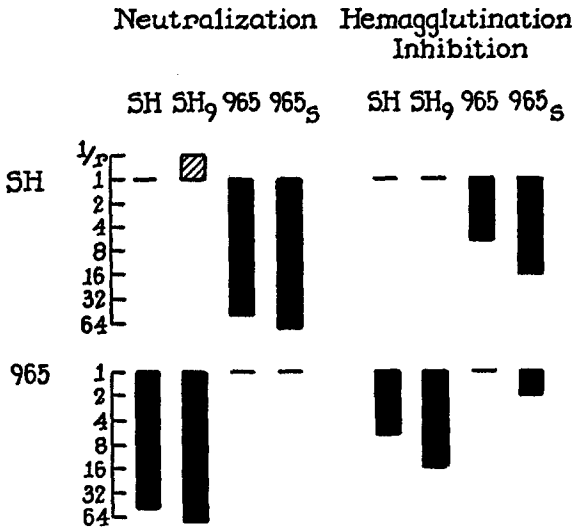

FIG. 5. Extent of *in ovo* and *in vitro* cross-serological reactions with SH, 965, and substrains obtained from them on serial passage *in ovo* in presence of heterologous immune serum. Serum titers and titer ratios are shown in Table X.

DISCUSSION

Influenza viruses appear capable of variation with respect to a number of properties and show evidence of variation with surprising frequency under a wide range of experimental conditions. Evidence of variation relative to pathogenicity for various species of laboratory animals, particularly ferrets (27), mice (28), hamsters (29), and chick embryos (18) has been obtained. Variation relative to the capacity to agglutinate erythrocytes of chickens and guinea pigs (30) also occurs and is especially marked with strains of influenza A virus (31). Variation affecting the rate of elution from erythrocytes has been demonstrated; such variants are obtained after treatment with physical (ultraviolet irradiation) or chemical (lanthanum acetate) agents (32). With a few strains strikingly abnormal variants pathogenic for the central nervous system have been obtained (33, 34). Among the array of properties which show variation with these infectious agents, that which is most arresting is variation in

antigenic pattern. Marked alterations in the antigenic structure of viruses raise problems of wide theoretical interest which also have obvious practical implications relative to identification, classification and immunization.

Variation in the antigenic constitution of influenza viruses may occur when a change in the host species is made and prolonged serial passage in a new host is carried out. There are indications that this may happen when chick embryos (18), embryo tissue cultures (16, 17), or mice (19, 21) are substituted for other host species. Strong evidence for antigenic variation on so called adaptation to the mouse lung has been assembled—sufficient to make it doubtful that the antigenic pattern of a mouse-adapted strain is a valid indication of the pattern of the original strain (19). As has been emphasized by Hirst (19) the comparison of the antigenic patterns of different mouse-adapted strains or of such strains with egg strains may lead to serious errors in assessing antigenic differences.

That variation in antigenic pattern also occurs on natural transmission of influenza viruses from man to man seems highly probable. This view is supported by earlier work (10) as well as by the evidence presented in this paper that strains of influenza A virus obtained in different years and maintained only in chick embryos for but a few passages show wide antigenic differences. That the small number of passages employed did not occasion the observed differences seems probable from the finding that, in numerous series, as many as 12 additional control passages in the embryo caused no demonstrable alteration in the antigenic pattern of any of 3 egg strains. This view is supported also by the now extensive evidence indicating that most egg strains obtained from a single epidemic are closely related (5, 9, 10) as well as by the fact that repeated recoveries in eggs of virus from a single patient yield strains which appear indistinguishable.

If the appearance of a variant with an antigenic pattern different from that of the original viral strain is the result of spontaneous variation analogous to the phenomenon of mutation in microbial species, it would be expected that the variant should replace the original strain whenever the host preferentially favored the multiplication of the variant. Moreover, it would be predicted that the antigenic pattern of the dominant variant should remain as a fixed and transmissible character until another variant arose which could replace the first. One means of favoring the selection of a variant of different antigenic pattern is to arrange conditions so that when a large number of viral particles are inoculated a large proportion are neutralized by antibody but those variant particles which react least with the antibody remain capable of multiplication. That such a procedure is effective and leads to the emergence of strains with antigenic patterns different from those of the parent strains appears evident from the results of this study.

The evidence indicates that strains which are related, to the extent that

they show appreciable cross-neutralization, yield antigenic variants on passage in the presence of heterologous immune serum. That such variants retain their new antigenic patterns on passage in the absence of a selective environment appears evident. How long the new character may persist remains to be determined. The apparent predictability of the antigenic pattern of the variant is not to be taken as an indication of a limited capacity of the virus to undergo variation but in all probability is simply a reflection of the specificity of the immune sera used to produce a selective environment. Evidence of predictable variation has been obtained previously with influenza viruses treated with either ultraviolet irradiation or lanthanum acetate (32) as well as with mumps virus on inhibition of multiplication with the capsular polysaccharide of Friedländer bacillus (35).

With influenza viruses a fixed non-varying antigenic constitution probably does not occur. An antigenic variant was obtained from PR8, which has undergone hundreds of passages in numerous species since its recovery in 1934, with as much ease as from any of the other strains which had been through only a few passages in a single host. It seems probable that by means of the procedure employed in this study antigenic variants could be obtained from any strain and that with different immune sera further variants could be selected from those which arise initially. Whether such a stepwise selection of variants would make possible the development of an A prime strain from a typical A strain or *vice versa* remains to be determined.

The fact that the antigenic dissimilarities between the original strains as well as between these strains and variants derived from them were both qualitatively and quantitatively different in cross-neutralization experiments as compared to cross-hemagglutination–inhibition experiments provides confirmatory evidence for the concept (25) that antibodies measurable by the *in vivo* technique are not identical with those measurable by the *in vitro* procedure.

SUMMARY

Antigenic variants of influenza A virus strains emerge on serial passage *in ovo* in the presence of immune serum against different but related strains. An old laboratory strain (PR8) which had been through hundreds of animal passages was as readily modified by this procedure as recently recovered strains. Such variants apparently can be obtained at will and show antigenic patterns which are reproducible and appear to be predictable in terms of the immune serum used for their selection. Variant strains retain their new antigenic patterns on serial passage *in ovo* in the absence of immune serum. Limited serial passage *in ovo* of strains in the absence of immune serum did not result in the emergence of antigenic variants. Similarly, serial passages of strains *in ovo* in the presence of immune serum against widely different strains, which

failed to show significant cross-neutralization, did not lead to the appearance of antigenic variants.

BIBLIOGRAPHY

1. Magill, T. P., and Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1936, **35**, 463.
2. Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.
3. Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.
4. Smith, W., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1938, **19**, 293.
5. Hirst, G. K., *J. Exp. Med.*, 1943, **78**, 407.
6. Friedewald, W. F., *J. Exp. Med.*, 1944, **79**, 633.
7. Magill, T. P., and Sugg, J. Y., *J. Exp. Med.*, 1944, **80**, 1.
8. Magill, T. P., and Sugg, J. Y., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 104.
9. Hirst, G. K., *J. Exp. Med.*, 1947, **86**, 367.
10. Taylor, R. M., *Am. J. Pub. Health*, 1949, **39**, 171.
11. Rasmussen, A. F., Stokes, J. C., and Smadel, J. E., *Am. J. Hyg.*, 1948, **47**, 142.
12. Kalter, S. S., Chapman, O. D., Feeley, D. A., and MacDowell, S. L., *J. Immunol.*, 1948, **59**, 147.
13. Hilleman, M. R., Mason, R. P., and Rogers, N. G., *Pub. Health Rep., U.S.P.H.S.*, 1950, **65**, 171.
14. Salk, J. E., and Suriano, P. C., *Am. J. Pub. Health*, 1949, **39**, 345.
15. Francis, T., Jr., Salk, J. E., and Quilligan, J. J., Jr., *Am. J. Pub. Health*, 1947, **37**, 1013.
16. Magill, T. P., and Francis, T., Jr., *J. Exp. Med.*, 1936, **63**, 803.
17. Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1943, **24**, 33.
18. Burnet, F. M., and Bull, D. R., *Australian J. Exp. Biol. and Med. Sc.*, 1944, 22, 173.
19. Hirst, G. K., *J. Exp. Med.*, 1947, **86**, 357.
20. Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 143.
21. Sugg, J. Y., *J. Bact.*, 1949, **58**, 399.
22. Rickard, E. R., Lennette, E. H., and Horsfall, F. L., Jr., *Pub. Health Rep., U.S.P.H.S.*, 1940, **55**, 2146.
23. Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.
24. Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.
25. Walker, D. L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1950, **91**, 65.
26. Burnet, F. M., and Lush, D., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18**, 49.
27. Francis, T., Jr., and Magill, T. P., *J. Exp. Med.*, 1935, **62**, 505.
28. Andrewes, C. H., Laidlaw, P. P., and Smith, W., *Lancet*, 1934, **2**, 859.
29. Friedewald, W. F., and Hook, E. W., Jr., *J. Exp. Med.*, 1948, **88**, 343.
30. Burnet, F. M., *Australian J. Sc.*, 1942, **5**, 81.
31. Burnet, F. M., Stone, J. D., Isaacs, A., and Edney, M., *Brit. J. Exp. Path.*, 1949, **30**, 419.
32. Björkman, S. E., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, **88**, 445.
33. Stuart-Harris, C. H., *Lancet*, 1939, **1**, 497.
34. Francis, T., Jr., and Moore, A. E., *J. Exp. Med.*, 1940, **72**, 717.
35. Ginsberg, H. S., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1949, **90**, 393.